

Protection against Carbon Tetrachloride-induced Hepatotoxicity by Pretreating Rats with the Hemisuccinate Esters of Tocopherol and Cholesterol

Marc W. Fariss,¹ Kay F. Bryson,¹ Elizabeth E. Hylton,¹ H. Robert Lippman,² Charles H. Stubin,¹ and Xiao-Guang Zhao¹

¹Environmental and Molecular Toxicology, Department of Pathology, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298-0662 USA;

²Department of Pathology, McGuire Veterans Administration Medical Center, Richmond, VA 23298-0662 USA

Previous studies have demonstrated that α -tocopheryl hemisuccinate (TS) protects hepatocyte suspensions from chemical-induced toxicity. It has been suggested that TS cytoprotection is related to unique properties of the TS molecule or is dependent on the cellular release and activity of unesterified α -tocopherol (T). To test the unique cytoprotective nature of TS *in vivo*, the protective ability of T and tocopherol esters against carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats was examined. Hepatoprotection [determined by serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels and histopathology] was not observed after T (or tocopheryl acetate and tocopheryl nicotinate) administration, even though this treatment resulted in a fivefold elevation in hepatic T content. Only pretreatment with TS (100 mg/kg, intraperitoneally) resulted in partial hepatoprotection against CCl_4 (2.9 g/kg, orally) toxicity. These findings suggest that hepatoprotection results not from the cellular accumulation of T but rather from the intact TS molecule. To test this hypothesis, the hepatoprotective capacity of cholesteryl hemisuccinate (CS), unesterified cholesterol, and cholesteryl acetate (CA) was examined against CCl_4 toxicity. As observed with the tocopherol derivatives, pretreatment with unesterified cholesterol or CA demonstrated no protective ability. However, when rats were pretreated with CS (100 mg/kg), the hepatotoxic effects of CCl_4 (elevated serum AST and ALT levels and centrilobular necrosis) were completely prevented. The prevention of CCl_4 -induced hepatotoxicity by CS and TS do not appear to result from an alteration in hepatic drug metabolism. These data clearly demonstrate that CS and TS are unique and powerful cytoprotective agents against CCl_4 hepatotoxicity *in vivo*. Furthermore, the protection observed is not the result of cellular T accumulation, but rather appears to depend on the hepatocellular accumulation of the intact TS molecule. **Key words:** carbon tetrachloride, cholesterol, cholesteryl hemisuccinate, hepatotoxicity, tocopherol, tocopheryl hemisuccinate, vitamin E. *Environ Health Perspect* 101:528–536(1993)

Numerous *in vitro* studies attest to the cytoprotective nature of α -tocopheryl hemisuccinate (TS). Incubation of TS with rat and canine (1) hepatocyte suspensions has been shown to protect these cells from the toxic effects of calcium ionophore A23187 (2), ethyl methanesulfonate (1,2), doxorubicin (2–4), hyperoxia (5), cadmium (6), ethacrynic acid (3) and tetrahydroaminoacridine (7). Carini et al. (8) demonstrated that TS, exogenously added to suspensions of rat hepatocytes and liver microsomes, consistently provided protection against ADP-iron-, CCl_4 - and cumene hydroperoxide-induced lipid peroxidation. Gogu and coworkers (9,10) demonstrated that normal murine bone marrow progenitor cells treated with TS were protected from the cytotoxic effects of zidovudine (AZT). Furthermore, Chinese hamster V-79 cells pretreated with TS were protected from chromate- (11) and ultraviolet-B light- (12) induced cytotoxicity. These findings suggest that TS-mediated cytoprotection is not selective for a particular chemical or toxic insult, cell type, or species. In fact, the diversity of these insults suggests that the observed cytoprotection may result from the ability of TS to intervene in the critical cellular events that lead to toxic cell death.

The investigators of the studies mentioned above suggest that TS cytoprotection results from the enhanced cellular release and accumulation of unesterified α -tocopherol (T), a membrane-bound antioxidant and free radical scavenger (2–4, 7–12). However, our studies using rat hepatocyte suspensions do not support this conclusion. Instead, we have demonstrated that incubation of hepatocytes with T or tocopheryl acetate (TA) results in elevated cellular α -tocopherol and TA levels, respectively, but does not protect cells from toxic injury (1,5,6). From these studies, we have concluded that TS cytoprotection is unique and depends on the cellular accumulation of the TS molecule and not T.

One possible explanation for the lack of protection afforded hepatocytes after T administration (1,4–6,8) is that exogenously added α -tocopherol, *in vitro*, is not properly incorporated into subcellular membranes at an active site or in an active configuration.

Attempting to exclude this possibility, we investigated the protective effect of *in vivo* TS administration (and other tocopherol analogs) on carbon tetrachloride-induced toxicity in rats. Furthermore, to assess the importance of the tocopherol molecule in cytoprotection, we also investigated the protective properties of another lipophilic succinate derivative, cholesteryl hemisuccinate (CS).

Materials and Methods

Di[tris(hydroxymethyl)-aminomethane]succinate (trizma succinate), tris(hydroxymethyl)aminomethane (trizma base), sodium succinate, cholesteryl acetate (CA; 99%), cholesteryl hemisuccinate, tris salt (99%, CS), dl- α -tocopheryl nicotinate (TN; 99%), 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone; 98+%) were purchased from Sigma Chemical Co., St. Louis, Missouri. Carbon tetrachloride (CCl_4 ; 99+%) and cholesterol (98%) were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin; d- α -tocopherol (T; 98%), d- α -tocopheryl hemisuccinate, free acid (TS; 99%), and d- α -tocopheryl acetate (TA; 99%) were gifts from The Henkel Corp., LaGrange, Illinois.

The method of Janoff et al. (13) was modified to prepare the tris salt of TS. Trizma base (7.5 mM or 913 mg) was dissolved in 25 ml of hot methanol. In a separate flask, TS (7.5 mM or 4.0 g) was dissolved in 25 ml of diethyl ether. Each solution was cooled to room temperature, mixed, and placed on a rotoevaporator under negative pressure. Once solvents were removed, we washed the resulting gelatinous white solid in diethyl ether (4 ml), stirred it, and allowed it to sit overnight. The ether was removed with a rotoevaporator and the resulting white solid dried in a desiccator containing calcium chloride. The melting point of the final product (4.5 g, 91% yield) was 88–91°C, and the purity was greater than 98+% as determined by acid/base titration of the amount of trizma base and TS, free acid present in the final product.

Address correspondence to M.W. Fariss, Department of Pathology, MCV/VCU, Richmond, VA 23298-0662 USA.

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For the experiments described in Tables 1–3, dosing solutions of T, TA, and TS free acid were prepared by dissolving the compounds in ethanol (2 parts) followed by peanut oil (3 parts) at a concentration of 100 mg/ml. In the experiments described in Tables 4 and 5, dosing solutions of cholesterol, CA, TS, and CS, tris salt were prepared at 25 mg/ml by dissolving the compounds in ethanol (1 part) followed by 3 parts olive oil (for cholesterol and CA) or polyethylene glycol 400 (for TS and CS). In the experiments described in Tables 6 and 7, dosing solutions (25 mg/kg) of TN, TS free acid and cholesterol were dissolved in olive oil (25 mg/ml), whereas the tris salts of TS and CS were fine suspensions (sonicated) of 25 mg/ml saline. The dosing solution of CCl_4 was dissolved in peanut oil (1 g/ml), and metyrapone was dissolved in corn oil (50 mg/ml). We prepared all dosing solutions immediately before administration.

Male Sprague-Dawley rats (175–275 g), given food and water *ad libitum*, were used for these experiments. Experimental animals were given an intraperitoneal (ip) injection or oral gavage of tocopherol analog (100 mg/kg body weight), cholesterol analog (100 mg/kg), or vehicle and fasted (as noted, several experiments used only fed rats). Twenty four hours later, each animal received CCl_4 (1.0 to 2.9 g/kg body weight) or peanut oil (vehicle) by oral gavage. One hour after CCl_4 administration, each animal received food and water *ad libitum*. We assessed hepatotoxicity 24, 48, or 72 hr after CCl_4 administration and monitored survival for 7 days in the acute toxicity experiments (Table 1).

Hepatotoxicity was determined by measuring serum AST and ALT levels and liver histopathology. Using ether anesthesia, we collected serum from the inferior aorta (at sacrifice) or from the tail vein at 24 or 48 hr after CCl_4 administration. We determined serum AST and ALT levels with a Roche Cobas Bio Clinical Analyzer using the spectrophotometric method adopted from Bergmeyer et al. (14).

For histopathology, we sacrificed experimental animals 48 or 72 hr after CCl_4 administration with ether anesthesia and exsanguination. The liver (or other tissues) was immediately removed, weighed, sliced into 2- to 3-mm sections, and fixed in 10% neutral buffered formalin. After 48 hr of fixation, tissues were paraffin infiltrated on a Miles Tissue-Tek VIP tissue processor. Four micron histologic sections were cut on a Reichert Histostat Rotary microtome and mounted on glass slides. All sections were stained with Harris hematoxylin and eosin (H&E) on a Shandon Varistain 24-3. In several experiments (described in Figure 2), liver tissue was cut

Table 1. Effect of tocopherol analog administration on the acute toxicity of CCl_4

Tocopherol analog administered ^a	Survival after CCl_4 treatment (g CCl_4 /kg)						Estimated LD ₅₀
	5.0	3.8	2.9	2.2	1.7	1.3	
Vehicle	ND	–	–	+	ND	ND	2.5
Tocopherol (100 mg/kg, ip)	ND	ND	–	–	–	+	1.5
Tocopheryl acetate (100 mg/kg, ip)	ND	–	–	+	ND	ND	2.5
Tocopheryl succinate (100 mg/kg, ip)	–	+	+	ND	ND	ND	4.4
Tocopheryl succinate (100 mg/kg, oral)	ND	–	–	+	ND	ND	2.5

^aTocopherol analogs and vehicle were administered and rats fasted for 24 hr before CCl_4 treatment. Survival is noted as + and results represent three to nine rats tested per treatment. LD₅₀ values were estimated by the up-and-down method of Dixon (15) and Bruce (16). ND, not determined.

into 1-mm cubes and fixed in 2% phosphate-buffered glutaraldehyde for 3 hr. After a phosphate buffer rinse, the tissues were postfixed 1 hr in 1% osmium tetroxide, dehydrated with ethanol, and embedded in Poly/Bed epoxy resin (Polysciences Inc., Warrington, Pennsylvania). Sections were cut 1 μm thick and stained with 1% toluidine blue. Histologic sections were examined (as described below) by a pathologist without knowledge of experimental treatment. To quantify CCl_4 -induced hepatocellular necrosis, each histologic liver section was rated for necrosis on a scale of 0–4 or was examined for the percentage of liver necrosis as measured with a Zeiss Videoplan system.

We examined multiple liver histologic cross-sections from each experimental animal and scored them as to the extent of liver necrosis using a scale of 0–4. The criteria for each score are as follows: 0 = no necrosis; 1 = necrosis limited to proximal perivenular (centrilobular) layers of hepatocytes without extending into the midzonal (mid-third between portal triad and terminal venule/central vein) region; 2 = necrosis of entire perivenular zone extending into the midzonal region, up to one-half the span of the hepatic lobule; 3 = necrosis of more than one-half of the hepatic lobule extending to within a few cell layers of the portal triad; and 4 = complete necrosis.

To determine the percentage of liver necrosis (% of liver), a histologic liver section was displayed on the screen of a Zeiss (Oberkochen, Germany) Videoplan monitor using a Hitachi CCTV camera mounted on a Zeiss light microscope with a 1 \times objective. Using the Videoplan digitizer tablet, stylus, and the data acquisition program, the Videoplan system calculated the total area of the liver section after drawing the outline of the liver. Next, the Videoplan system calculated the percentage of liver necrosis (% of total liver showing necrosis) after drawing the outline of the

necrotic areas in the liver section. For each experimental liver, we examined two liver sections; each section was measured twice, and a mean value was calculated from these four determinations. A tissue control and a constructed square control were examined before analyzing experimental samples. Using a square with sections of 20, 40, 60, 80, and 100% drawn on graph paper as a template, the accuracy of the Videoplan in determining the area of each section (expressed as a % of the total area) with a 2% error was confirmed daily. Using a liver section control (from a CCl_4 -treated rat), the accuracy and reproducibility of the Videoplan and its operator were confirmed daily. We analyzed this control liver section for percent liver necrosis on five separate occasions with a mean and SE of 52.7 \pm 2.2%. Before analyzing experimental samples, we determined the percent necrosis in the liver control section within 2 SEs of the calculated mean value (48–57%).

To determine the effect of tocopherol analog administration on the acute toxicity of CCl_4 , we estimated the LD₅₀ by a modified up-and-down method of Dixon (15) and Bruce (16). In brief, the dose of CCl_4 administered was adjusted by a factor of 1.3 until successive lethal and nonlethal doses were found. The true LD₅₀ value for each treatment is assumed to fall between these successive CCl_4 doses with opposite effects. The initial dose was 2.9 g CCl_4 /kg, and survival was monitored for at least 7 days after CCl_4 treatment to ensure recovery.

Tissue concentrations of T, TA, and TS were determined by the HPLC method of Fariss et al. (1). Liver, brain, kidney, heart, and lung were prepared for analysis by a freeze-clamp technique (immediately frozen with liquid N_2 , pulverized to a powder with a mortar and pestle, and stored at -80°C). For analysis, 25–50 mg of powdered tissue was weighed (or 100 μl of plasma or blood added) in a microcentrifuge tube and treated as described for

viable hepatocytes in the previous method (1). We determined the tissue concentration of T from the initial tissue extraction, and the T ester concentration (TS or TA) was determined by measuring the amount of T released from base hydrolysis of the first extract (1). A standard curve for each tocopherol analog was analyzed with each set of experimental samples. The limit of detection for T, TA, and TS by this method is 10 nmol/g tissue and 5 nmol/ml plasma or blood.

We investigated the effect of TS and CS on microsomal drug metabolism using the pentobarbitone (PB) sleeping-time test (17). Male Sprague Dawley rats (160–200 g) were given an intraperitoneal injection of vehicle (olive oil); TS in olive oil (100 mg/kg); cholesterol in olive oil (64 mg/kg) plus tris succinate in saline (59 mg/kg); or CS, tris salt in saline (100 mg/kg) plus olive oil (same dose as C treatment) and fasted for 24 hr. Next, PB (50 mg/kg, intraperitoneally) was administered to each rat, and the sleeping time was recorded. The absence of the animal's righting reflex was used to indicate sleep. At the start of sleeping time, we placed the animal in the supine position, and the sleeping time ended once the animal assumed a prone position. As a control, an inhibitor of cytochrome P-450, metyrapone (100 mg/kg) or its vehicle (corn oil) was administered intraperitoneally to rats 30 min before PB (50 mg/kg), and the sleeping time was determined. The pretreatment regimen of each animal was unknown to the investigator during the sleeping time determination.

Data were analyzed for significance ($p < 0.05$) using single-factor analysis of variance (ANOVA) and Scheffe's multiple comparison test (StatView II for Macintosh, version 1.04, Abacus Concepts Inc., Berkeley, California). The Student's *t* test (unpaired) was used to determine the significant difference ($p < 0.05$) between the two treatments in each PB sleeping time experiment.

Results

The estimated LD₅₀ determined for CCl₄ was 2.5 g/kg in rats receiving vehicle pretreatment (Table 1). Rats pretreated with a single dose of T or TA (100 mg/kg) before an oral gavage of CCl₄ were not afforded protection against the lethal effects of this chlorohydrocarbon. In fact, the administration of unesterified T potentiated CCl₄ acute toxicity (estimated LD₅₀ of 1.5 g/kg). In contrast, rats pretreated with TS (100 mg/kg) were protected against CCl₄-mediated toxicity as demonstrated by a 76% increase in the estimated LD₅₀ to 4.4 g/kg (Table 1). Interestingly, when TS was administered orally, no pro-

Table 2. Effect of tocopherol analog administration on CCl₄ hepatotoxicity

Tocopherol analog administered ^a	Serum enzyme concentration			Tissue concentration		
	AST (10 ³ U/l)	ALT (10 ³ U/L)	Liver necrosis (% of liver)	Liver	Fat	
Vehicles (no CCl ₄)	0.07 ± 0.01	0.03 ± 0.00	0	T T ester	48 ± 2 ND	42 ± 13 ND
Vehicle (+ CCl ₄ , 2.9 g/kg)	15.60 ± 5.11	2.82 ± 1.65	57.7 ± 8.9	T T ester	51 ± 4 ND	66 ± 14 ND
Tocopherol (+ CCl ₄ , 1.7 g/kg)	17.33 ± 7.22	4.68 ± 0.29	62.4 ± 3.5	T T ester	60 ± 9 ND	397 ± 80** ND
Tocopheryl acetate (+ CCl ₄ , 2.9 g/kg)	20.22 ± 3.52	6.11 ± 0.42	50.9 ± 7.2	T TA	85 ± 3 ND	76 ± 8 ND
Tocopheryl succinate (+ CCl ₄ , 2.9 g/kg)	3.43 ± 1.50*	0.63 ± 1.17*	28.7 ± 0.9**	T TS	117 ± 15* ND	124 ± 16 47 ± 20

^aTocopherol analogs and vehicle were administered (100 mg/kg, ip) 24 hr before a single oral dose of CCl₄. Rats were fasted 24 hr before CCl₄ administration. Liver necrosis, tissue T, and T ester levels and serum AST and ALT levels were determined in specimens obtained 48 hr after CCl₄ treatment. Values are the means ± SEM ($n = 3-4$). ND, not determined.

* $p < 0.05$, as compared to other CCl₄ treatments.

** $p < 0.001$, as compared to other CCl₄ treatments.

Table 3. Tissue concentrations of tocopherol and tocopherol esters, 24 and 48 hr after tocopherol analog administration

Tocopherol analog administered		Tissue concentration (nmol/g) ^a						
		Liver	Brain	Kidney	Heart	Lung	Blood	Plasma
Vehicle	T	28 ± 3	27 ± 1	35 ± 5	27 ± 5	47 ± 1	10 ± 1	13 ± 1
24 hr after	T ester	ND	ND	ND	ND	ND	ND	ND
Tocopherol (100 mg/kg, ip)	T	128 ± 10*	32 ± 2	41 ± 2	36 ± 3	70 ± 6	16 ± 1	20 ± 2
	T ester	ND	ND	ND	ND	ND	ND	ND
Tocopheryl acetate (100 mg/kg, ip)	T	56 ± 10	34 ± 5	35 ± 1	43 ± 4	54 ± 3	22 ± 1	18 ± 1
	TA	92 ± 6	ND	30 ± 8	37 ± 2	74 ± 4	7 ± 2	6 ± 2
Tocopheryl succinate (100 mg/kg, ip)	T	51 ± 5	34 ± 3	25 ± 1	31 ± 2	42 ± 2	13 ± 0	14 ± 2
	TS	119 ± 16	ND	31 ± 6	47 ± 3	90 ± 1	7 ± 1	8 ± 1
Tocopheryl succinate (100 mg/kg, oral)	T	66 ± 12	42 ± 4	39 ± 2	62 ± 2	63 ± 4	16 ± 1	14 ± 1
	TS	ND	ND	ND	ND	ND	ND	ND
Vehicle	T	38 ± 6	34 ± 3	35 ± 3	47 ± 5	54 ± 9	11 ± 2	9 ± 1
48 hr after	T ester	ND	ND	ND	ND	ND	ND	ND
Tocopheryl succinate (100 mg/kg, ip)	T	66 ± 6	nd	39 ± 4	38 ± 10	88 ± 3	19 ± 2	20 ± 1
	TS	30 ± 53	nd	ND	ND	ND	ND	ND

^aValues are the means ± SEM ($n = 3-4$). ND, not detected; nd, not determined.

* $p < 0.001$, as compared to other tocopherol analog treatments.

tection was observed. Histological examination of rat tissues (liver, lung, kidney, and heart), obtained 48 hr after a lethal dose of CCl₄, demonstrated extensive periportal and midzonal liver necrosis, but histopathology was unremarkable for the other tissues examined. Liver histology was normal in TS-pretreated rats that survived 7 days after CCl₄ treatment.

The protective effect of TS administration against CCl₄-induced hepatotoxicity is shown in Table 2 and Figure 1. Each rat received an approximate LD₅₀ dose of CCl₄ (2.9 g CCl₄/kg for all treatments except 1.7 g/kg for T-pretreated rats). Rats pretreated with vehicle demonstrated a massive increase in liver-associated serum

enzymes AST (15,600 U/l) and ALT (2,820 U/l), 48 hr after CCl₄ administration. In addition, approximately 58% of the liver was found to be necrotic in each animal from this treatment group (Table 2, Fig. 1B). Pretreatment of rats with T or TA did not significantly alter the hepatotoxic effect of CCl₄ treatment as indicated by serum enzyme levels, percent liver necrosis, and histopathology (Table 2, Fig. 1C). These findings are in agreement with the acute toxicity data in that comparable lethal doses of CCl₄ given to TA- and T-pretreated rats resulted in similar hepatotoxic damage. In contrast, rats pretreated with TS were significantly protected from CCl₄ hepatotoxicity, with serum AST and

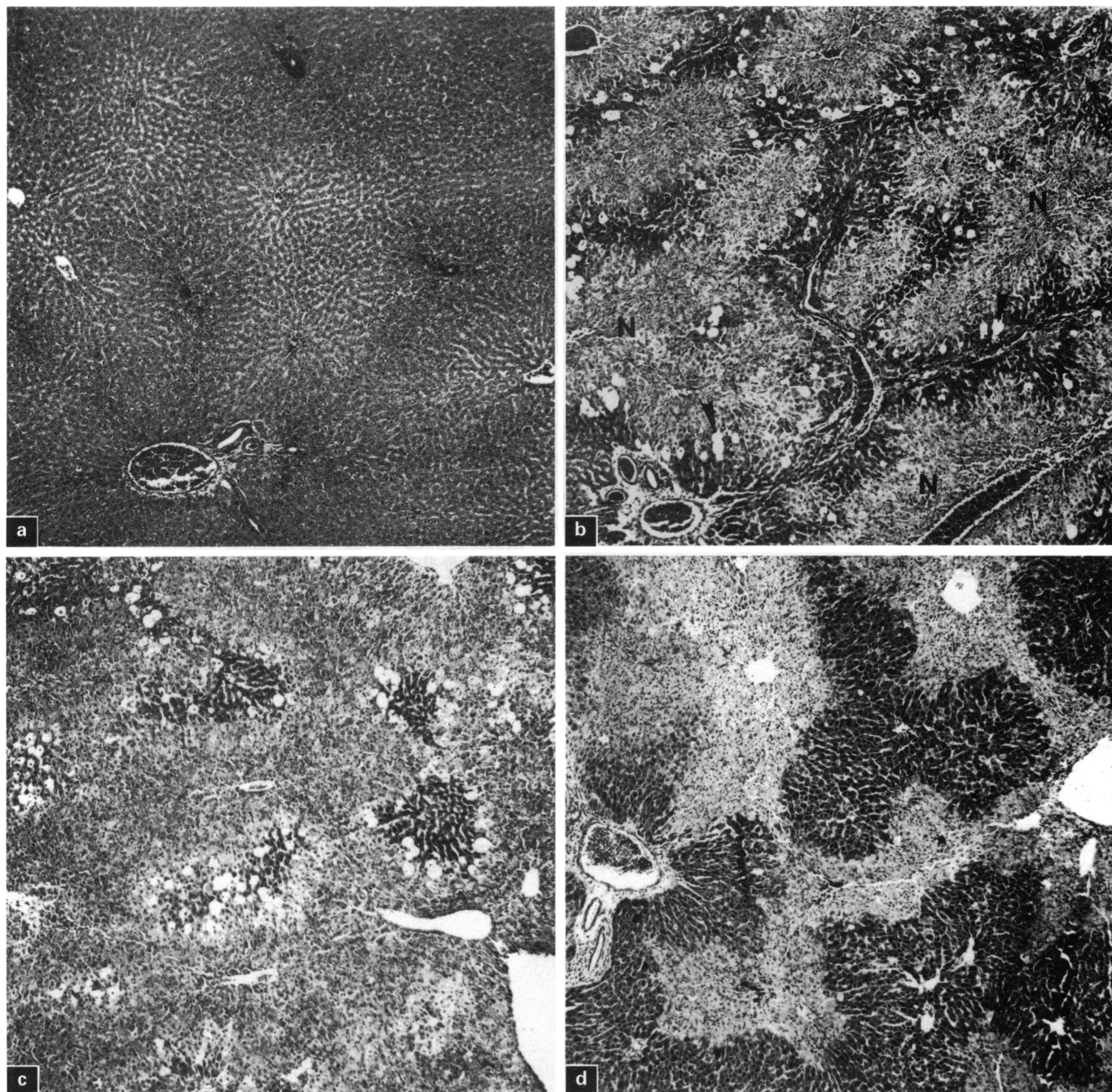


Figure 1. Light micrographs of paraffin-embedded rat liver. All tissues were stained with hematoxylin and eosin. (A) Representative section from rat treated with vehicle, no pathologic changes present. (B) Representative section from rat treated with CCl_4 (2.9 g/kg); necrosis (N) of all hepatocytes except those within a few cell layers of the portal tracts (necrosis score 3). There is ballooning degeneration at the interface (arrowheads). (C) Representative sections from rat treated with CCl_4 (1.7 g/kg) and T; identical pathologic changes as that observed in panel B (necrosis score 3). (D) Representative section from rat treated with CCl_4 (2.9 g/kg) and TS; necrosis of perivenular and part of midzonal hepatocytes (necrosis score 2). The periportal and proximal midzonal hepatocytes are preserved with little degeneration. All photographs are $50\times$.

ALT levels of 3430 and 630 U/l, respectively (77% reduction as compared to CCl_4 only treatment). Administration of the cytoprotective agent TS also significantly reduced CCl_4 -mediated hepatic necrosis by 50% from 58% to 29% (Table 2) and appeared to limit the extent of damage (ballooning degeneration) to periportal and proximal midzonal hepatocytes (Fig. 1D). Hepatic T levels in TS-treated rats and fat T levels in T-treated rats were significantly elevated as compared to the

other treatment groups 48 hr after CCl_4 treatment. Tocopherol esters were not detected in the liver 48 hr after CCl_4 treatment.

The tissue (liver, brain, kidney, heart, lung, whole blood, and plasma) concentration of T and tocopherol esters (TA and TS) were measured 24 hr after tocopherol analog administration (no CCl_4), and the results are shown in Table 3. This time course (24 hr after) was used to determine the tissue (liver) concentration of T, TA,

and TS at the time of CCl_4 exposure. Rats pretreated with T for 24 hr demonstrated a fivefold increase in the amount of unesterified T in the liver (128 nmol/g) as compared to vehicle-treated rats (28 nmol/kg). However, this T pretreatment did not result in protection. A twofold increase in liver T concentration was observed with TA and TS pretreatments, but only the administration of TS resulted in protection against CCl_4 toxicity and hepatic TS accumulation (Table 3). Thus, TS-mediated

protection appears to depend on the hepatocellular accumulation of TS. This conclusion was confirmed by our finding that the oral administration of TS eliminates both protection and liver and tissue accumulation of TS (Table 3). Furthermore, the tissue distribution results in Table 3 demonstrate that 24 hr after a single dose of TS (100 mg/kg), a significant ($p < 0.05$ versus vehicle control) accumulation of TS was found in all tissues examined, except the brain. However, 48 hr after TS administration, tissue TS levels were not detectable or were not significantly different from vehicle control.

To investigate the importance of the tocopherol molecule in cytoprotection, we also examined the protective properties of another lipophilic hemisuccinate derivative, CS, as well as unesterified cholesterol

and CA. Data from these studies (Table 4) clearly demonstrate that CS tris salt is a powerful protective agent against the hepatotoxic effects of a sublethal dose of CCl_4 (1 g/kg). In fact, CS pretreatment completely protected rats from hepatotoxicity at 24, 48, and 72 hr after CCl_4 exposure as measured by serum ALT levels and hepatic necrosis. However, cholesterol or CA pretreatment did not significantly protect rats from the hepatotoxic effects of CCl_4 administration (Table 4). A histological examination of the hepatic terminal venules (Fig. 2) revealed that treatment with 1 g CCl_4 /kg results in perivenular necrosis and collapse (necrosis score 1) with large globule fatty degeneration of midzonal hepatocytes (fat globules turn black with osmium postfixation; Fig. 2B). In contrast, CS pretreatment prevented CCl_4 -induced

perivenular necrosis (necrosis score 0) and dramatically reduced the fatty degeneration of hepatocytes (Fig. 2C).

To determine the influence of fasting on the cytoprotective capacity of TS and CS, rats were pretreated with TS, CS, tris salt, or cholesterol plus sodium succinate plus trizma base and fed for 24 hr before CCl_4 administration (2 g/kg). Data from these studies (Table 5) demonstrate that CS tris salt pretreatment completely protected fed rats from hepatotoxicity at 24, 48, and 72 hr after CCl_4 exposure as measured by serum ALT levels and hepatic necrosis. However, TS and cholesterol plus succinate plus trizma pretreatments did not afford rats significant hepatoprotection. Interestingly, fed rats given 2 g CCl_4 /kg demonstrated less hepatic damage with greater variability (ALT levels) within treatment groups than fasted rats receiving 1 g CCl_4 /kg (Table 4). In both fed and fasted rats, liver damage (as measured by ALT levels) was observed predominantly at 24 and 48 hr after CCl_4 exposure, but peak enzyme leakage varied within a treatment group between these two time points. Though serum ALT levels returned to near control levels by 72 hr after CCl_4 treatment, hepatic necrosis was still detectable by histopathology at this time point (Tables 4 and 5).

Because of the striking protective capacity of CS tris salt, the protective abilities of the free acid and tris salt of TS were compared with CS tris salt and TN pretreatment against a sublethal hepatotoxic dose of CCl_4 (1 g/kg). The results of this study, shown in Table 6, indicate that the administration of TN and TS free acid did

Table 4. Effect of cholesterol analog administration on CCl_4 -induced hepatotoxicity

Cholesterol analog administered ^a	Serum ALT (10^3 U/L)			Hepatic necrosis score (0–4)
	24 hr	48 hr	72 hr	
Vehicles (no CCl_4)	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0 ± 0
Vehicle (+ CCl_4)	0.55 ± 0.14	0.92 ± 0.14	0.10 ± 0.01	1 ± 0
Cholesterol (+ CCl_4)	1.45 ± 0.34	1.16 ± 0.41	0.11 ± 0.04	1 ± 0
Cholesteryl acetate (+ CCl_4)	0.83 ± 0.38	0.96 ± 0.32	0.11 ± 0.04	1 ± 0
Cholesteryl succinate tris salt (+ CCl_4)	0.04 ± 0.01*	0.02 ± 0.0*	0.02 ± 0.0*	0 ± 0

^aCholesterol analogs and vehicle were administered (100 mg/kg, ip) 24 hr before a single oral dose of CCl_4 (1.0 g/kg). Rats were fasted 24 hr before CCl_4 administration. Serum ALT levels were determined at 24, 48, and 72 hr after CCl_4 treatment. The liver necrosis score was determined from specimens obtained 72 hr after CCl_4 treatment. Values are the means ± SEM ($n = 3$ –5).

* $p < 0.01$, as compared to other CCl_4 treatments.

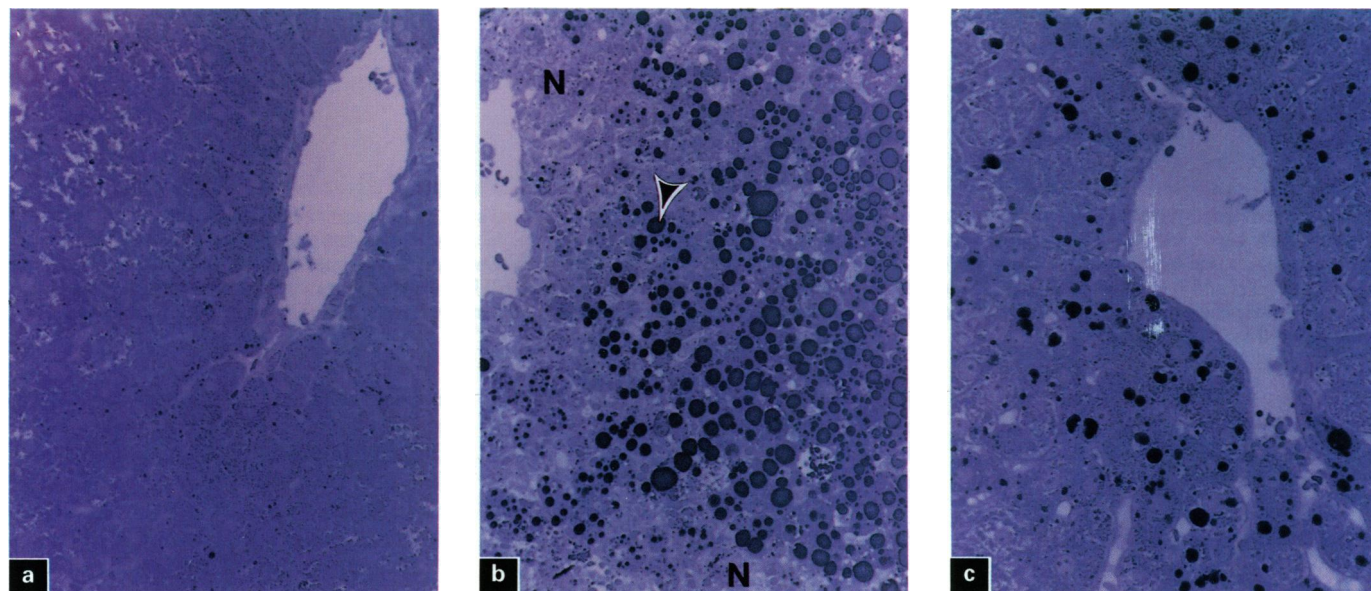


Figure 2. Light micrographs of plastic-embedded rat liver, postfixed in osmium tetroxide and stained with toluidine blue. (A) Vehicle control (saline, no CCl_4): hepatic terminal venule showing normal hepatocytes with infrequent and small fat globules (black). (B) CCl_4 -treated group (1 g/kg): hepatic terminal venule with perivenular necrosis (N) and collapse with large globule fatty degeneration (arrowhead) of midzonal hepatocytes, (necrosis score 1). (C) CCl_4 (1 g/kg) and CS-treated group: hepatic terminal venule with ballooning and fatty degeneration of perivenular hepatocytes with occasional hepatocellular necrosis or dropout. Midzonal cells, at periphery, are intact with infrequent fat globules (necrosis score 0). All photographs are 280 \times .

Table 5. Effect of tocopherol and cholesterol analogs on CCl₄-induced hepatotoxicity in fed rats

Cholesterol analog administered ^a	Serum ALT (10 ³ U/L)			Hepatic necrosis score (0–4)
	24 hr	48 hr	72 hr	
Vehicles (no CCl ₄)	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0 ± 0
Vehicle (+ CCl ₄)	0.26 ± 0.13	0.39 ± 0.28	0.08 ± 0.04	0.5 ± 0.5
Tocopheryl succinate (+ CCl ₄)	0.32 ± 0.34	0.53 ± 0.38	0.07 ± 0.04	0.75 ± 0.5
Cholesterol + tris + succinate (+ CCl ₄)	0.31 ± 0.17	0.48 ± 0.24	0.10 ± 0.04	0.75 ± 0.5
Cholesteryl succinate, tris salt (+ CCl ₄)	0.05 ± 0.02*	0.03 ± 0.01*	0.03 ± 0.0*	0 ± 0

^aTocopherol and cholesterol analogs and vehicle were administered (100 mg/kg, ip) 24 hr before a single oral dose of CCl₄ (2.0 g/kg). Rats were fed before CCl₄ administration. Serum ALT levels were determined at 24, 48, and 72 hr after CCl₄ treatment. The liver necrosis score was determined for specimens obtained 72 hr after CCl₄ treatment. Values are the means ± SEM (*n* = 4–5).

**p* < 0.01 as compared to vehicle (+CCl₄).

Table 6. Effect of tocopherol and cholesterol analogs on CCl₄-induced hepatotoxicity

Analog administered ^a	Serum enzyme concentration		Hepatic necrosis score (0–4)
	AST (10 ³ U/L)	ALT (10 ³ U/L)	
Vehicles (no CCl ₄)	0.11 ± 0.03	0.05 ± 0.01	0 ± 0
Vehicle (olive oil) (+ CCl ₄)	4.41 ± 2.05	1.95 ± 0.71	2.0 ± 1.0
Tocopheryl nicotinate (+ CCl ₄)	4.95 ± 1.38	2.12 ± 0.52	ND
Tocopheryl succinate, free acid (+ CCl ₄)	2.06 ± 0.53	1.08 ± 0.23	1.25 ± 0.50
Tocopheryl succinate, tris salt (+ CCl ₄)	0.79 ± 0.26*	0.52 ± 0.16*	1.0 ± 0.0
Cholesteryl succinate, tris salt (+ CCl ₄)	0.14 ± 0.02**	0.06 ± 0.01**	0.25 ± 0.50

^aTocopherol and cholesterol analogs and vehicle were administered (100 mg/kg, ip) 24 hr before a single oral dose of CCl₄ (1.0 g/kg). Rats were fasted 24 hr before CCl₄ administration. Serum AST and ALT levels and liver necrosis were determined in specimens obtained 48 hr after CCl₄ treatment. Values are the means ± SEM (*n* = 3–5). ND, not determined.

**p* < 0.01 as compared to vehicle (+ CCl₄).

***p* < 0.001 as compared to vehicle (+ CCl₄).

Table 7. Effect of tocopheryl succinate and cholesteryl succinate administration on pentobarbitone (PB) sleeping time in the rat

Experiment	Pretreatment ^a	Sleeping time (min)
1	Vehicle (corn oil)	120 ± 9
	Metyrapone (100 mg/kg, ip)	900 ± 120*
2	Vehicle (olive oil)	72 ± 14
	Tocopheryl succinate, free acid (100 mg/kg, ip)	86 ± 1
3	Cholesterol (64 mg/kg, ip) and tris succinate (59 mg/kg, ip)	117 ± 30
	Cholesteryl succinate, tris salt (100 mg/kg, ip)	148 ± 40

^aThe pretreatment compounds were administered and rats fasted for 24 hr before a single dose of PB (50 mg/kg, ip). Metyrapone or vehicle (corn oil) were given 30 min before PB. Values are the means ± SEM (*n* = 3–5).

not afford rats significant protection against the hepatotoxic effects of CCl₄. The percent protection [as determined by ALT values with negative (no CCl₄) and positive (+CCl₄) controls assumed to be 100% and 0% protection] for TN and TS free acid were 0% and 46%, respectively. In contrast, the administration of the tris salt form of TS provided highly significant hepatoprotection (75% protection). How-

ever, the tris salt of CS proved to be the most effective protective agent tested, providing virtually complete protection against CCl₄-mediated hepatotoxicity.

To assess the effect of TS and CS pretreatment on hepatic drug metabolism, the influence of these cytoprotective agents on pentobarbitone-mediated sleeping time in the rat was determined (Table 7). The pretreatment of rats with metyrapone, a

known inhibitor of cytochrome P-450-mediated drug metabolism, resulted in a 650% increase in PB sleeping time. However, a significant increase in PB sleeping time was not observed in rats pretreated with TS free acid (19% increase) or CS tris salt (26% increase) as compared to vehicle controls and nonprotective conditions (cholesterol and tris succinate).

Discussion

Because exposure to CCl₄ results in extensive cellular lipid peroxidation, numerous investigators have examined the protective role of cellular α-tocopherol, the predominant membrane-bound antioxidant and free radical scavenger, in CCl₄-induced hepatotoxicity. Attempts to supplement endogenous stores of cellular T, however, have resulted in conflicting reports on the cytoprotective abilities of vitamin E administration against CCl₄ toxicity. The published reports range from protection (18–20) and no effect (20–22) to potentiation (20,23) of CCl₄ toxicity after vitamin E administration. The present study demonstrates distinct differences in the cytoprotective nature of various tocopherol analogs. As measured by survival, liver-associated serum enzyme levels, and histopathology, protection against CCl₄ hepatotoxicity was not observed after pretreating rats (24 hr) with a single dose (100 mg/kg) of unesterified T or the acetate and nicotinate esters of T. Thus, the supplementation of hepatic T levels (fivefold increase) in a presumably physiologically active configuration (*in vivo* administration and disposition) failed to protect rats from the toxic effects of CCl₄. In fact, we found that T administration enhanced the toxic effect of CCl₄, while TA and TN administration did not alter CCl₄-mediated toxicity. The variable findings, reported here and in numerous other reports, on the protective abilities of vitamin E administration (18–23), illustrate the need for additional studies on the influence of vitamin E dosing regimens on T subcellular disposition and chemical-induced toxicity. These studies are necessary to understand and have confidence in the use of T or other tocopherol analogs as a therapeutic strategy for CCl₄ or other chemical poisonings.

In contrast to T, TA, and TN, the administration of the hemisuccinate ester of T protected rats from the lethal and hepatotoxic effects of 2.9 g CCl₄/kg body weight and resulted in a significant accumulation of hepatocellular TS. These data support the results and conclusions from our previous *in vitro* studies, demonstrating that TS is a unique and effective cytoprotective agent. Our finding that TS administered orally results in no hepatic accumulation of TS (presumably due to

TS hydrolysis by gut esterases, as tissue T levels are elevated in the absence of tissue TS; Table 3) and no protection against CCl_4 toxicity also supports our hypothesis that the presence of cellular TS is essential for cytoprotection against CCl_4 toxicity. Interestingly, the protection observed for TS free acid in rats treated with a lethal dose of CCl_4 (2.9 g/kg) was not observed when the concentration of CCl_4 administered was reduced to a sublethal dose in fasted and fed rats. Though TS free acid pretreatment did reduce CCl_4 -induced hepatotoxicity by 46% (in fasted rats) when sublethal concentrations of CCl_4 were administered (Table 6), the degree of protection was not significant ($p < 0.05$) as determined by ANOVA using Scheffe's multiple comparison test. Wolfgang et al. (21) have also reported the modest protective abilities of TS free acid administration against a sublethal dose of CCl_4 . However, the administration of the tris salt of TS did result in significant hepatoprotection ($p < 0.01$). An explanation for the apparent difference in the cytoprotective abilities of the free acid and the tris salt of TS is unknown. We speculate that differences in hydrophilicity between these two forms of TS might influence the bioavailability of TS after administration and hence explain the variability in cytoprotective ability. This hypothesis is in agreement with Carini and co-workers' (8) explanation for enhanced protection against CCl_4 -induced lipid peroxidation with the water-soluble form of TS, TS polyethylene glycol ester, as compared to TS free acid.

One possible explanation for the unique cytoprotective abilities of TS is that the subcellular distribution of TS (as compared to T administration) is distinctive, and thus T released from TS at this novel subcellular site is essential for protection. In support of this hypothesis, we found that TS pretreatment resulted in hepatic T levels 48 hr after CCl_4 treatment that were significantly higher than levels observed for nonprotective vitamin E pretreatments (even though these pretreatments increased adipose T levels by 10-fold; Table 2). These findings indicate a unique ability of TS to maintain hepatocellular T levels during a toxic insult or they may merely be the result, rather than the cause, of cytoprotection (cellular T is lost after cell death). We support the later explanation, but this question can only be answered *in vitro*, by separating viable from nonviable hepatocytes before analysis during a toxic CCl_4 insult. To argue against a novel cellular disposition for TS, our tissue distribution data, measured 24 hr after a single dose of T ester, does not show a significant difference in the disposition of TS and TA.

As shown in Table 3, similar concentrations of TS and TA were found in each tissue examined. Furthermore, the release of T as an explanation for TS cytoprotection does not appear to be the critical event because the substitution of the tocopherol portion of the TS molecule with cholesterol results in a compound (CS) with remarkable protective properties against CCl_4 -induced hepatotoxicity. Thus, our studies clearly demonstrate that the release of tocopherol is not essential for the cytoprotection observed. Because protection was not observed after cholesterol and CA administration, and cholesterol has no reported antioxidant and free radical scavenging abilities, we again conclude that the protection observed results from the cellular accumulation and the unique properties of CS and TS. The protection against CCl_4 hepatotoxicity observed with CS administration was striking, with complete protection against CCl_4 -induced necrosis and a dramatic reduction in fatty degeneration as demonstrated by light microscopy. These results indicate that CS pretreatment not only maintained cell viability during a toxic insult with CCl_4 but also maintained hepatic function (processing of lipid) during this insult. Like TS, the tris salt of CS is a more effective cytoprotective agent than the free acid form (personal observation). Again, we speculate that the insoluble nature of CS free acid appears to preclude its protective abilities.

As we are the first to describe CS and TS cytoprotection *in vivo*, little is known about the mechanism of TS and CS protection against CCl_4 -mediated hepatotoxicity. One possible explanation is that TS and CS pretreatments simply delay the onset of the toxic response rather than provide true protection. This possibility is unlikely because we have shown that TS protection against the lethal effects of CCl_4 continues for at least 7 days with no liver histopathology observed at that time. In addition, using both histopathology and serum ALT levels, we demonstrated that CS completely protected fed and fasted rats from the hepatotoxic effects of CCl_4 for 72 hr (a period of time in which serum AST levels, for CCl_4 -treated rats without protection, have nearly returned to normal endogenous levels). It is important to point out the advantages of using both liver histopathology and serum ALT levels to assess hepatotoxicity. We have found, in agreement with previous reports, that the time course of hepatic AST leakage after CCl_4 treatment can vary significantly from animal to animal in a treatment group. Whereas the leakage of cellular enzymes is a transient event, liver necrosis is evident for 3–5 days. Thus, by determining the extent of liver necrosis (score) 48 or 72 hr

after a CCl_4 insult, in combination with serum enzyme levels, we gain considerable confidence in reporting the degree of hepatotoxicity or hepatoprotection.

Because the hepatic metabolism of CCl_4 is essential for the expression of CCl_4 toxicity, one obvious explanation for this protection would be the inhibition of drug metabolism by CS and TS administration. Previous reports have demonstrated that numerous drugs that potentiate or inhibit CCl_4 toxicity similarly affect the metabolism of pentobarbitone, resulting in a decrease or increase in sleeping time, respectively (24–27). As our studies demonstrated no significant alteration in pentobarbitone sleeping time after CS or TS pretreatment, we conclude that inhibition of CCl_4 metabolism probably is not responsible for the cytoprotection observed with these compounds. However, additional studies examining the effect of these protective agents on hepatic P-450 IIE1 activity and spectral P-450 levels are required.

Another possible explanation for the unique protective properties observed after TS and CS pretreatment is the release of succinate from hepatocellular TS or CS. Previous studies indicate that the administration of succinate can protect mitochondria, cells, and organisms from a variety of toxic insults (28–30) and that succinate is a preferred mitochondrial substrate with respect to energy production (oxidative phosphorylation) and the reduction of pyridine nucleotides (31,32). Because the cellular uptake and accumulation of succinate is severely limited by its hydrophilicity (33), the administration of large concentrations of succinate (g/kg body weight, *in vivo*) are required to alter the cellular energy status or provide cytoprotection. In contrast, the administration of TS or CS provides a lipophilic carrier for succinate, thus promoting both cellular uptake and accumulation. Interestingly, Simon et al. (34) reported that the majority of intravenously administered TS was found as intact TS in the hepatic microsomal and mitochondrial fractions. In the present study, we found that approximately 120 nmol succinate/g liver is released from hepatocellular TS during a 24-hr period (the hepatic TS concentration at 24 and 48 hr after TS administration were 120 nmol/g and 0 nmol/g, respectively; Table 3). Assuming that the normal endogenous succinate concentration in fasted rat liver is 170 to 270 nmol/g (35,36), the amount of succinate released from hepatocellular TS would result in a 45–70% increase in succinate levels over a 24-hr period. This substantial increase in succinate, released and used in the hepatocyte, might enable the maintenance of cellular metabolic and

repair processes required for survival during a toxic CCl_4 insult. Additional experimental evidence will be required to confirm the role of succinate in TS and CS protection against CCl_4 -induced hepatotoxicity. Because an analytical method to measure tissue CS concentrations has not yet been developed, we can only assume (due to common physicochemical properties) that the hepatic accumulation and disposition of CS is similar to that found for TS.

A final explanation for TS- and CS-mediated hepatoprotection is that the amphipathic TS and CS molecules are the protective agents. Data from the present study suggest that the ionic nature of TS or CS is important for protection. If the acidic nature of the tocopherol or cholesterol molecule is eliminated, as seen with T, TA, TN, C or CA, the protective properties of these compounds are eliminated. Similar findings were also reported in our previous *in vitro* studies on TS-mediated cytoprotection (1,5,6). Once in the cell, the TS and CS molecules might alter membrane stability or function by interacting with membrane-bound proteins and/or with the unsaturated fatty acid portion of phospholipid (tocopherol or cholesterol portion) as well as with the polar region of the phospholipid (ionic succinate moiety) (37). In fact, a unique membrane-associated property of TS and CS (not found with other nonionic cholesterol or tocopherol analogs) is their ability to spontaneously form multilamellar liposomes (bilayers) in aqueous solution (13). However, this bilayer organization of TS can be disrupted by increasing the calcium concentration to 2.5 mM or greater (38). This calcium concentration (>2.5mM) also eliminated TS-induced protection against toxic injury in isolated hepatocytes (1).

Numerous reports indicate that TS and CS administration do indeed stabilize membranes (13,37,39–41) and alter membrane enzymatic and receptor activity (40–45). For example, Brase and Westfall (42) reported that the addition of TS stimulates rat liver phenylalanine hydroxylase activity, while Chelliah and Fariss (44) have recently discovered that both TS and CS are potent inhibitors of purified electric eel-derived acetylcholinesterase activity but not butyrylcholinesterase activity. Numerous reports also attest to the effect of CS on the physical state of cellular membranes (increased viscosity), which can influence a variety of membrane functions (39–41). For example, Levy et al. (39) demonstrated a marked reduction in cellular ion leakage after human erythrocyte membranes were enriched with CS. Kolena and Kasal (40) reported that the incubation of rat testicular membranes with CS and other dicar-

boxylic acid esters of cholesterol resulted in an increase in both membrane lipid microviscosity and in human chorionic gonadotropin (hCG)-receptor binding. The elimination of the free carboxylic acid group of CS and the other cholesterol esters abolished their stimulatory effect on both the leutenizing hormone/hCG receptor and membrane microviscosity. Using synaptosomal brain membranes, Lazar and Medzihradsky (41) demonstrated that the addition of CS restored the microviscosity and opioid receptor binding capacity of membranes fluidized by fatty acids. It is well known that numerous physiological and pathological conditions, including CCl_4 toxicity (46–48), can modulate the lipid composition and fluidity of biological membranes resulting in diminished structural integrity and altered membrane-bound enzyme activity (e.g., calcium ATPase activity). We speculate that the activity of CS and TS on cellular membranes, as described above, might prevent or reverse the membrane solvent effect (49) and lipid peroxidative (46–48) effect associated with CCl_4 hepatotoxicity.

In summary, we have found that supplementation of endogenous unesterified T levels does not protect the liver from the toxic effects of CCl_4 . In contrast, the administration of the succinate esters of tocopherol and cholesterol afford rats protection against CCl_4 -induced toxicity. In fact, rats pretreated with CS were completely protected from CCl_4 -mediated liver damage. The observed protection appears to depend on the hepatocellular accumulation of TS and CS and does not appear to be related to an alteration in hepatic drug metabolism. Future investigations on the mechanism of CS and TS protection against CCl_4 toxicity should provide valuable information about the critical cellular events responsible for CCl_4 toxicity and potential strategies to protect ourselves from this toxic chemical and other chlorohydrocarbons.

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